

MOLECULAR COMPLEMENTARITY BETWEEN NUCLEAR DNA AND ORGAN-SPECIFIC CHROMOSOMAL RNA*

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We have shown earlier¹ that chromosomes contain RNA which is associated with the chromosomal proteins, the majority of which are histones. In pea chromatin these RNA molecules are approximately 40 nucleotides in length, contain about 27 mole per cent dihydrouridylic acid, and are associated with chromosomal DNA in such a way that they are protected from attack by RNase. As a first step in the study of the role of this class of RNA we have investigated the characteristics of its hybridization with nuclear DNA. It is shown below that the RNA component of protein-bound chromosomal RNA is heterogeneous and that it hybridizes with 5 per cent of nuclear DNA, a ratio approximating the ratio of this RNA to DNA in native chromatin.

Materials and Methods.—Preparation of chromosomal RNA: Uniformly P³²-labeled pea seedlings were produced as described earlier (Huang and Bonner²) by soaking 5-kg lots of pea seeds (var. *Alaska*) in solution containing 50 mc carrier-free H₃PO₄ adjusted to pH 7.5 with 1 M tris. The soaked seeds, which fully imbibe the P³²-containing solution, were planted in vermiculite and germinated for 6 days in the dark at 25°C. The apical buds were then removed, each preparation yielding approximately 800 gm fresh weight of buds. From these, chromatin was prepared by the method of Huang and Bonner.² The buds were homogenized (Waring blender, full speed, 60 sec) in a grinding medium consisting of sucrose, 0.25 M; MgCl₂, 0.001 M³ and tris buffer, 0.05 M, pH 8.0. The resulting homogenate was then filtered successively through cheesecloth and Miracloth and centrifuged for 30 min at 4,000 × *g*. The pelleted crude chromatin was resuspended and washed 5 times by pelleting from 0.01 M tris, pH 8.0, each time for 10 min at 10,000 × *g*. Although in early experiments the resulting crude chromatin was further purified by sucrose density gradient centrifugation (Huang and Bonner²), such purification was not found to influence the properties of the final product RNA. In general, therefore, the final crude chromatin pellet was dissolved in 4 M CsCl and centrifuged for 20 hr at 40,000 rpm in the no. 40 Spinco rotor. Under these conditions the DNA pellets and the chromosomal proteins float to the surface of the CsCl solution, carrying the protein-bound RNA with them. The protein skins thus formed were next removed and washed 5 times by pelleting from 70% EtOH.

The chromosomal proteins were next suspended in 2 ml of pronase (2 mg/ml in 0.01 M tris, pH 8.0) which had been previously autodigested for 90 min at 37°C to remove nucleases. After incubation for 8–10 hr at 37°C any remaining insoluble protein was centrifuged off, the supernatant made 0.2 N in potassium acetate, and RNA precipitated by the addition of 2 volumes of 95% EtOH. The precipitated RNA was dissolved in a small volume of 0.1 M NaCl, 7 M urea, and 0.01 M tris, pH 8.0. The sample was then chromatographed on an A-25 DEAE Sephadex column as described by Hall *et al.*,³ eluting with a linear gradient of NaCl, 0.1–1.1 M, all in 7 M urea, and 0.01 M tris, pH 8.0. The principal UV-absorbing component, eluting at approximately 0.55 M NaCl, was collected, dialyzed against two changes of distilled water, lyophilized, and the RNA then again precipitated from 0.2 N KAc by 2 volumes of EtOH. The resulting material was used for the studies described below. RNA so prepared was rendered totally acid-soluble by hydrolysis with boiled pancreatic RNase or by KOH hydrolysis (0.3 N, 18 hr at 37°C) and possessed an E_{260 mμ} of 8.55 × 10³ on the basis of phosphorus analysis.

Preparation of transfer and ribosomal RNA: For the preparation of pea transfer and pea ribosomal RNA the supernatant remaining from the first pelleting of the chromatin was further centrifuged at 30,000 × *g* for 30 min. The resulting supernatant was made to 0.05 M NaAc, 2.5% butanol, 0.5% SLS, and 5 × 10⁻⁴ M MgCl₂, and extracted 3 times with cold, buffer-saturated phenol. The whole cytoplasmic RNA, freed of phenol by ethanol precipitation and dialysis, was

next subjected to treatment with DNase (Worthington electrophoretically purified) and again phenol-extracted to free it of the enzyme. Ribosomal and transfer RNA were then separated on a methylated albumin Kieselguhr (MAK) column, using a gradient of NaCl. The purified transfer RNA was treated with exonuclease I to free it of all contaminating DNA, as described by Ofengand *et al.*⁴ Alkali-stable material in the final preparation was always less than 0.05%. Purity of the preparations was also checked by sucrose density gradient centrifugation.

For preparation of whole cytoplasmic RNA, the same procedure was employed with the exception that the initial phenol extraction was done at 60°C.

Preparation of messenger RNA: For comparison of the properties of freshly transcribed messenger RNA with those of chromosomal RNA, pea chromatin purified by centrifugation through sucrose was incubated in standard RNA polymerase reaction mixture¹⁴ containing 8-C¹⁴-ATP. After incubation (30 min, 37°C) 2 volumes of 95% EtOH were added, the chromatin and freshly synthesized RNA were pelleted by centrifugation, and the pellet was washed 5 times with 70% ethanol. The final pellet was dissolved in 4 M CsCl and treated as described under preparation of chromosomal RNA.

Preparation of DNA: Chromatin of pea buds, prepared as outlined above, but not P³²-labeled, was dissolved in 4 M CsCl and centrifuged for 20 hr at 40,000 rpm in the Spinco no. 40 rotor. The resulting DNA pellet was subjected to two successive Sevag treatments according to the method of Marmur⁵ and then wound out from 70% EtOH. The DNA, redissolved in dilute saline citrate (0.015 M NaCl, 0.0015 M sodium citrate) was next treated with boiled RNase (40 µg/ml) for 1 hr at 37°C after which the RNase was removed with pronase which had been predigested to remove nucleases. The DNA was then again treated by the Sevag procedure, wound out from 70% EtOH, and dialyzed against dilute saline citrate. The pea DNA thus prepared possessed an $E_{260\text{ m}\mu}$ of 7.33×10^3 based on phosphorus analysis and is free of detectable RNase activity. DNA prepared as above but with Sevag treatment of pea chromatin rather than CsCl centrifugation as the initial step appears to be identical in properties.

Preparation of filters: Pea DNA prepared as described above was denatured by alkali, the denaturation being monitored by UV absorbance. The denatured DNA was applied in $6 \times \text{SSC}$ (SSC is 0.15 M NaCl, 0.015 M sodium citrate) to Schleicher and Schuell B6 nitrocellulose filters, 25 mm in diameter, exactly as described by Gillespie and Spiegelman.⁶ After application of DNA, the filters were washed in $6 \times \text{SSC}$, dried at room temperature in air for 4 hr, and further dried for 2 hr at 80°C in a vacuum oven. Filters were similarly prepared but without applied DNA for determination of background.

Retention of DNA by filters: It has been our experience that when amounts of DNA less than about 25 µg per filter are applied as described above, more than 98% of the DNA is retained by the filter. However, DNA is steadily lost from the filters during incubation in $2 \times \text{SSC}$ at 66°C. We have found that with incubation times of 24 hr, only 75% of the DNA originally contained on our filters is retained. We therefore routinely monitor the amount retained at the end of the hybridization and washing procedures by hydrolysis of DNA from the filter (5% HClO₄, 10 min, 100°C) and spectrophotometry.

Hybridization: Hybridization was carried out in $2 \times \text{SSC}$, two filters in 2 ml of solution, and at a temperature of 66°C for a time period in general of 20 hr. The filters were then washed, treated with RNase, rewashed, dried, and counted in a Packard Tri-Carb scintillation counter, all according to the procedures of Gillespie and Spiegelman.⁶ All RNA samples for hybridization were heated to 90°C for 10 min in 0.01 M tris (pH 8.0) and then quick-cooled immediately before use. This treatment is necessary to achieve full hybridization of chromosomal RNA.

Results.—Hybridization of ribosomal and transfer RNA: For the standardization of our procedures we have first determined the extent of hybridization at saturation of pea ribosomal RNA with pea DNA, a matter previously investigated by Chipchase and Birnstiel.⁷ The data of Table 1 show that by the present procedures whole pea ribosomal RNA (combined 28S and 18S species) hybridizes at saturation with approximately 0.26 per cent of whole pea DNA, a number in satisfactory agreement with the 0.28 per cent determined by Chipchase and Birnstiel. The data of Table 1 show that in similar experiments the transfer RNA of pea plants hybridizes

TABLE 1
HYBRIDIZATION OF PEA BUD RIBOSOMAL AND TRANSFER RNA TO PEA DNA

Species of DNA	Species of P ³² -RNA	Unlabeled competing RNA	Ratio of competing to P ³² -RNA	DNA hybridized at saturation (%)
Pea	Pea ribosomal	None	—	0.26 ± 0.009
Pea	Pea transfer	None	—	0.057 ± 0.004
Pea	Pea ribosomal	Pea ribosomal	2	0.097
Pea	Pea ribosomal	Pea transfer	1.5	0.24
Pea	Pea transfer	Pea transfer	2	0.021
Pea	Pea transfer	Pea ribosomal	1.5	0.054

to an extent of approximately 0.057 per cent with pea DNA at saturation. This value is higher than, but of the same order of magnitude as, the 0.025 per cent found for *E. coli* by Goodman and Rich⁸ and by Giacomoni and Spiegelman⁹ and the 0.015 per cent found for *Drosophila* by Ritossa, Atwood, and Spiegelman.¹⁰ The hybridization of pea transfer RNA with pea DNA is not influenced by the presence of an excess of unlabeled pea ribosomal RNA, and vice versa. That the expected competition for hybridization between labeled and unlabeled RNA's of the same type does occur is established below. Hybridization of neither transfer nor ribosomal RNA was influenced by prior melting and quick cooling of the RNA.

Characteristics of the RNA of protein-bound chromosomal RNA: The chromosomal RNA isolated by the procedure outlined under *Methods* elutes from DEAE Sephadex as a single, sharp peak and at a NaCl concentration of 0.55 M as shown in Figure 1. It is followed by a second smaller peak of nucleic acid eluting at 0.65 M NaCl. The material of the second peak is, however, DNA. The material eluting at a NaCl concentration of 0.55 M, like the same class of RNA hydrolyzed while still associated with chromosomal protein,¹ is rich (26 mole %) in dihydrouridylic acid. Content of this base was determined both by separation of P³²-labeled nucleotides on a Dowex 1 × 8 column as previously reported,¹ and by chemical determination of the ureido group content of the alkaline hydrolysate of the RNA by the method of Shepherdson and Pardee.¹¹ The chain length of the purified RNA as determined by the end group method is 42, in agreement with our earlier determination. Further properties of the purified chromosomal RNA include a sedimentation coefficient (determined by analytical band centrifugation¹²) of 3.2, its total conversion to

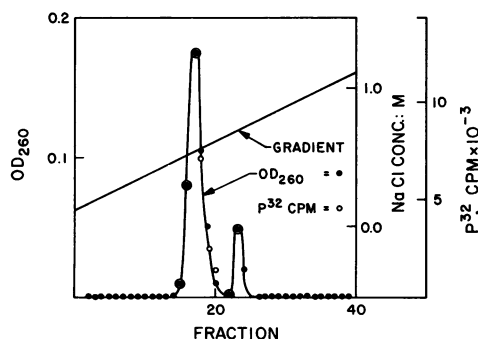


FIG. 1.—Elution profile of pea bud chromosomal RNA from DEAE-Sephadex column. Eluting solvent consists of a gradient of NaCl, 0.1–1.1 M, all in 7 M urea.

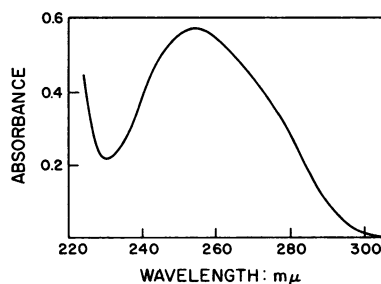


FIG. 2.—Spectrum of purified pea bud chromosomal RNA.

TABLE 2
PURIFIED RNA OF HISTONE-RNA IS RENDERED COMPLETELY ACID-SOLUBLE BY
HYDROLYSIS EITHER BY RNASE OR BY KOH

Treatment	Input	OD ₂₆₀	Acid-sol.	Acid-soluble (%)
None*	0.54		0.006	1.1
KOH, 0.3 N†	0.62		0.63	100.0
RNase‡	0.50		0.49	98.0

* All samples made 5% in HClO₄ at end of treatment and OD₂₆₀ of supernatant was determined.

† RNA samples treated with 0.3 N KOH for 18 hr at 37°C.

‡ RNA sample treated with 45 µg RNase for 1 hr at 37°C.

acid-soluble material by KOH hydrolysis or RNase treatment (Table 2), and its spectrum (Fig. 2).

The chromosomal RNA differs from messenger RNA generated by transcription of chromatin by RNA polymerase in its short chain length and unusual base composition.¹ The two classes of RNA also behave differently when chromatin is centrifuged in 4 M CsCl. Protein-bound chromosomal RNA accompanies protein and floats. Messenger RNA, on the contrary, pellets. To establish this point, purified chromatin was incubated with the four riboside triphosphates, one C¹⁴-labeled, in the presence of *E. coli* RNA polymerase. The resulting RNA, together with the chromatin template freed of reaction mixture by repeated pelleting from 70 per cent EtOH, was next dissolved in 4 M CsCl and centrifuged just as for the preparation of protein-bound chromosomal RNA. RNA generated by transcription of pea chromatin, which has been shown to possess messenger RNA activity (Bonner, Huang, and Gilden¹³), does not remain associated with chromosomal protein during centrifugation in 4 M CsCl, but pellets as is shown in Table 3.

Hybridization of chromosomal RNA: The data of Figure 3 show that chromosomal RNA hybridizes at saturation with approximately 5 per cent of nuclear DNA. At this level of hybridization and the concentration of RNA required to just achieve it, approximately 2.5 per cent of the RNA present in the 2-ml reaction mixture is hybridized.

The data of Table 4 show that the hybridization of pea bud chromosomal RNA to pea nuclear DNA is specific. This class of RNA does not hybridize with DNA of HeLa, rat, cauliflower, or lambda DNA.¹⁵ Table 4 also includes data on specific activities of RNA used, background of the assay, and number of counts actually hybridized.

Neither transfer nor ribosomal RNA competes with chromosomal RNA for hybridization to nuclear DNA, as is shown in Table 5.

That the RNA is not degraded during the hybridization procedures was shown by experiments in which the RNA-DNA hybrid was melted in the presence of 1 per cent formaldehyde, quick-cooled, and subjected to sucrose density gradient

TABLE 3
DISTRIBUTION OF NEWLY FORMED mRNA AFTER CENTRIFUGATION IN 4 M CsCl

	Amount	Per cent of total
Amt. in surface skin, accompanying chromosomal protein	10.3 mµmoles	3.0
Amt. in pellet, accompanying DNA	337.0 mµmoles	97.0

Purified chromatin, 20 OD₂₆₀, was incubated for 30 min at 37°C in 7.5 ml of standard reaction mixture.¹⁴ The chromatin together with the product RNA formed by transcription of the chromatin *in vitro* (see *Methods*) were then precipitated with 2 volumes of EtOH, washed 5 times by reprecipitation from 70% EtOH, and then dissolved in 4 M CsCl and centrifuged for 16 hr at 35,000 rpm in Spinco SW-39 rotor. Nucleic acids pellet while chromosomal protein forms a surface skin. These were separated, washed in 70% EtOH, dissolved, and counted.

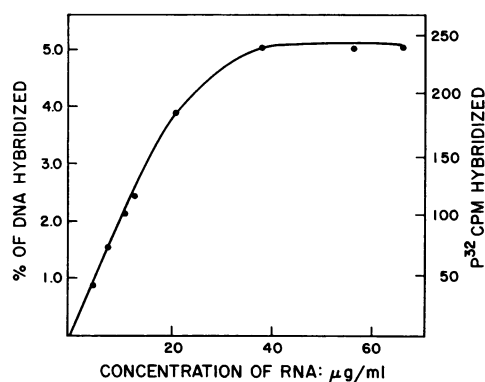


FIG. 3.—Hybridization of pea nuclear DNA by pea bud chromosomal RNA as a function of RNA concentration. Hybridization for 24 hr at 66°C in $2 \times \text{SSC}$.

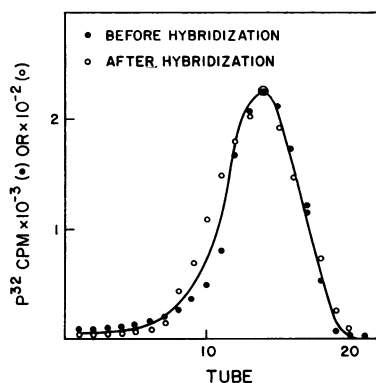


FIG. 4.—Sucrose density gradient (5–20% in 0.01 *M* tris pH 8.0, 1% formaldehyde) of pea bud chromosomal RNA, before and after hybridization to pea nuclear DNA. After hybridization, the hybridized material was heated for 10 min, 100°C, in 0.01 *M* tris pH 8.0 in the presence of 1% formaldehyde and then quick-cooled.

centrifugation. The data of Figure 4 show that the peak sedimentation velocity of the once-hybridized RNA is identical with that of the original unhybridized material.

Competition between chromosomal RNA of different organs: The extent of the similarities of chromosomal RNA's of different organs of the same organism may be investigated by hybridization-competition. The data of Figure 5 show again that unlabeled pea bud chromosomal RNA competes with P^{32} -unlabeled pea bud chromosomal RNA for hybridization to nuclear DNA. Unlabeled chromosomal RNA from chromatin of developing pea cotyledons also competes with the pea bud RNA.¹⁶ However, it is less effective in this function than is pea bud RNA itself.

Chromosomal RNA confined to the nucleus: In order to determine whether the nucleotide sequences characteristic of chromosomal RNA are shared by any RNA molecules in the cytoplasm, whole cytoplasmic RNA was allowed to compete with chromosomal RNA for hybridization to nuclear DNA. Figure 6 shows that there is no competition between unlabeled cytoplasmic RNA and labeled chromosomal RNA even when very large excesses of the former are used. Similar results were obtained when unlabeled pea bud chromosomal RNA was allowed to compete against labeled whole cytoplasmic RNA (which hybridized with 4.4% of the nuclear DNA at saturation). We conclude that the sequences characteristic of chromosomal RNA are largely or entirely confined to the nucleus.

TABLE 4
CHARACTERISTICS OF THE HYBRIDIZATION OF THE RNA OF PEA CHROMOSOMAL PROTEIN RNA TO VARIOUS DNA'S

DNA Source of DNA	on Filter Amt. of DNA (μmoles)	P ³² -RNA		RNase-Resistant Counts			DNA hybridized at saturation (%)
		$\mu\text{Moles/ml}$	Cpm/ml	Background (no DNA on filter)	on DNA filter	Net counts	
Pea	0.13	0.117	4,180	4.3	237.3	233.0	5.0
Lambda	0.11	0.117	4,180	4.1	5.5	1.4	0.0
Cauliflower	0.080	0.117	1,810	2.1	4.1	2.0	0.02
Rat	0.12	0.234	6,820	10.8	9.7	—	0.0
HeLa	0.047	0.234	6,820	10.8	8.5	—	0.0

TABLE 5
COMPETITION CHARACTERISTICS OF THE HYBRIDIZATION OF PEA BUD CHROMOSOMAL
RNA TO PEA DNA*

P^{32} -RNA	Competing unlabeled RNA	DNA hybridized (%)
Chromosomal RNA, 0.117 μ mole/ml	None	5.05
Chromosomal RNA, 0.025 μ mole/ml	None	1.56
Chromosomal RNA, 0.025 μ mole/ml	Transfer RNA, 0.050 μ mole/ml	1.75
Chromosomal RNA, 0.025 μ mole/ml	Ribosomal RNA, 0.050 μ mole/ml	1.71

* All RNA's are derived from pea.

Discussion.—Histone-associated RNA of pea chromatin hybridizes with a substantial proportion of the pea genome. Pea bud nucleohistone contains approximately 8 per cent as much RNA as DNA (Huang and Bonner¹), and approximately one half of this is recovered associated with histone after banding of nucleohistone in CsCl. The proportion of the genome hybridized by the purified RNA thus approximates the ratio of the RNA to DNA in the original chromatin. Since hybridization of chromosomal RNA to nuclear DNA is not influenced substantially by the presence of whole cytoplasmic RNA, the sequences characteristic of chromosomal RNA must be confined to the nucleus. Chromosomal RNA resembles in this respect the nuclear RNA of Harris *et al.*,¹⁶ which turns over rapidly within, but does not appear to escape from, the nucleus. Even more parallel may be found in the nuclear RNA of Shearer and McCarthy¹⁷ which is not only confined to the L-cell nucleus, but in addition hybridizes with *ca.* 4.4 per cent of nuclear DNA. Chromosomal RNA with properties generally similar to that of peas has been found, using methods identical to those used with peas, in the chick embryo,¹⁸ rat ascites tumor,¹⁹ and rat liver.²⁰

What can be the role of a class of RNA which is on the one hand associated with characteristic chromosomal protein and on the other hand capable of hybridizing with a relatively large proportion of the genome? The fact that the sequences of the RNA molecules of different organs are in part different from one another suggests that these molecules may be concerned with gene expression.

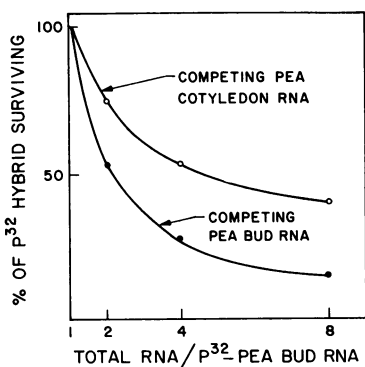


FIG. 5. — Hybridization-competition between P^{32} -labeled pea bud chromosomal RNA and unlabeled pea bud or pea cotyledon chromosomal RNA. Concentration of P^{32} -labeled pea bud chromosomal RNA 37.5 μ g/ml throughout.

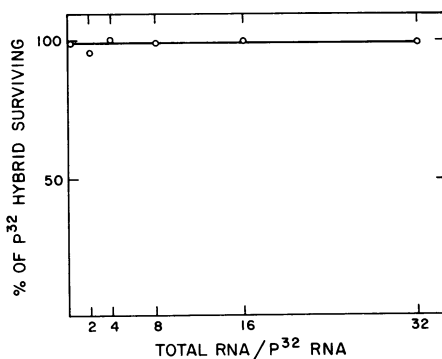


FIG. 6. — Hybridization-competition between P^{32} -labeled pea bud chromosomal RNA and unlabeled whole pea bud cytoplasmic RNA. Concentration of P^{32} -labeled pea bud chromosomal RNA 37.5 μ g/ml throughout.

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